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Amyotrophic lateral sclerosis and the innate immune system

Protocol for establishing a biobank and statistical analysis plan

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Abstract

Introduction: Amyotrophic lateral sclerosis (ALS) is a devastating, progressive disease that causes degeneration of the motor neurons leading to paresis of the bulbar and the skeletal musculature. The pathogenesis of ALS remains unknown. We will test the hypothesis that the complement system is involved in the pathophysiology of ALS. This protocol article describes our Danish ALS biobank project. The primary aim of the project is to obtain biological material from ALS patients for the current study as well as for future studies.

Methods and analysis: The project is a prospective, observational case-control study. The participants are ALS patients, neurologically healthy controls, and non-ALS neurological controls. Each participant consents to be interviewed and to donate blood and cerebrospinal fluid to the biobank. Analysis of the complement system will be carried out on the three groups of patients and compared.

Ethics and dissemination: The project has been approved by the Committees on Health Research Ethics in the Capital Region of Denmark (Approval number H-16017145) and the Danish Data Protection Agency (File number 2012-58-0004). All results will be published in peer-reviewed, medical journals and presented at scientific conferences.

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Keywords: Amyotrophic lateral sclerosis, complement system, bio-bank, protocol, innate immune system, case-control study

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Article summary

Strengths and limitations of this study

- This prospective, observational study ensures a national ALS biobank which will facilitate many future clinical studies of a rare disease such as ALS.
- Even though ALS is a heterogenous disease, all patients face rapid deterioration and severe distress and the design of this study provides recruitment of ALS patients at an early clinical stage hence facilitating trial participation.
- The diagnosis of ALS is difficult, and patients follow highly variable courses of disease; thus some patients who have been recruited for the study will eventually be diagnosed with other diseases and need to be excluded from the present study; conversely, some patients who are in the early phase of ALS may be missed because of atypical symptoms and will therefore be recruited in a later phase.

Introduction

Amyotrophic lateral sclerosis (ALS) is an aggressive disease that causes progressive degeneration of the upper and lower motor neurons leading to severe muscular dystrophy, fasciculations, hyperreflexia, and paresis of the bulbar and skeletal musculature. As the disease inevitably progresses, the patient becomes unable to move, speak, swallow, and breathe. In addition to the neuromuscular symptoms, frontotemporal dementia appears in up to 15 % of all ALS patients. [1]

ALS is a rare disease with an incidence of 1-2/100.000 and a prevalence of 4-6/100.000. The median survival time is 24-48 months from onset of symptoms. [2]

Five to ten per cent of all ALS cases are of a familiar type (fALS) which is genetically heterogeneous with a Mendelian inheritance pattern. Another group is spontaneous (sALS) with no family history of ALS. [3]

The pathogenesis of ALS remains unknown and presently, there is no effective treatment to stop the progression. The immune system has been hypothesized to be an important, pathophysiological factor. This is based on the fact that immunological reactions appear both in the proximity of the degenerating motor neurons in the central nervous system (CNS) and systemically. [4]

The complement system plays a central role in the innate immune system. The complement system initiates inflammation and activates innate as well as adaptive immune cells by detecting invading pathogens or damaged host cells. According to recent animal models of ALS, the very first detectable pathophysiological changes are seen in the neuromuscular junctions and might be a sign of aberrant complement activation. [5 6] Such inappropiate activation of the complement cascades may participate in the initiation of the destruction of the neuromuscular junctions by an autoinflammatory mechanism. Furthermore, aberrant complement activation has been found in the proximity of the degenerating motor neurons during ALS progression. In addition, findings of complement activation in the blood and in the muscle tissue, as well as an unexplained erythrocyte cytotoxicity phenomenon found in the plasma of ALS patients, suggest that the complement system could play an important role in the pathophysiology during the progression of ALS. [4 7]

To be able to develop an effective medical treatment, it is essential to study the pathogenesis and the pathophysiology of ALS. Since complement inhibition

employing the terminal complement inhibitor ravulizumab now is entering a phase 3 clinical trial for the treatment of ALS, it is crucial to firmly establish how the complement system affects the progression of ALS (https://news.alexionpharma.com). Understanding the pathophysiology of the disease provides a tool to develop diagnostic methods such as biomarkers. This may optimise the diagnostic process ensuring a faster and more precise diagnosis. In addition, this could increase the knowledge of the heterogeneity of the ALS symptoms.

Aim

The overall, long-term objective of this project is to establish an ALS bio-bank to facilitate future basic ALS research. Under the assumption that ALS is an autoinflammatory disease, another aim of the project is to disclose whether complement activation plays an essential role in the pathophysiology of ALS. The protocol describes the design and data collection methods in detail in order to inform other researchers of the bio-bank.

Methods

Design

This Danish national ALS bio-bank project is designed as a prospective, observational case-control study, based on the SPIRIT guidelines. The study comprises four substudies (Clinical trial 1-4, Table 1).

Participants

The national ALS bio-bank comprises samples from three groups of patients: ALS patients, neurologically healthy patients (healthy controls), and patients

with neurological diseases other than ALS (neurological controls). All the participants included are above 18 yrs.

Patients with ALS*:* Patients referred to an ALS out-patient clinic at either of five Danish hospitals (Table 2) due to symptoms suspected to be early symptoms of ALS are informed and invited to participate in the project during the standard clinical workup. If a patient at the end of the planned workup is diagnosed with either probable or definite ALS according to the El Escorial Revised criteria [8] and consents to inclusion, the patient is included in the project. Furthermore, *all probable or definite ALS patients* who have already been permanently associated to an ALS outpatient clinic (post clinical investigation) and who accept to undergo an additional lumbar puncture are invited to be part of the project.

Neurologically healthy controls: Neurologically healthy patients scheduled for elective orthopaedic surgery under spinal anaesthesia are invited to participate.

Neurological controls: These participants are recruited among patients admitted to hospital due to acute neurological symptoms that require a lumbar puncture as part of the clinical investigation, or who are referred for a planned lumbar puncture as part of a clinical neurological investigation due to neurological symptoms.

Exclusion criteria: Subjects with motor neuron disease, chronic inflammatory, or autoimmune diseases are excluded from the neurologically healthy control group and the neurological control group.

Settings

ALS patients are recruited from five ALS out-patient clinics at five Danish neurological departments: Bispebjerg Hospital, Rigshospitalet (Glostrup) (both

University of Copenhagen), Roskilde University Hospital, Odense University Hospital, and Aarhus University Hospital.

Neurologically healthy controls are recruited at Gildhøj Private Hospital, Denmark.

Controls from the neurological group are recruited at the neurological department at Rigshospitalet (Glostrup), Denmark. At each hospital, one local investigator is appointed. During sample collection, the primary investigator is present to obtain biological material from each patient and to process and freeze the samples using a portable "mini-lab". All data collected on case report forms are anonymised, gathered, and stored in an audited, central, electronic database. All biological material is transported to the Department of Clinical Immunology at Rigshospitalet, Denmark to be stored in bio-banking facilities.

Data collection

In ALS patients and patients in the neurological control group, blood samples are drawn from a peripheral vein after the performance of the lumbar puncture. In neurologically healthy controls, for logistical reasons, the blood samples are drawn before the lumbar puncture.

All the samples are processed by the primary investigator at the respective inclusion site and are transported in a - 20 °C freezer to a central bio-bank where they are stored at - 80 °C. (Figure 1)

Biological samples

Blood samples

Venous blood samples are collected by the primary investigator or by a skilled clinical nurse. Blood is collected in

Sample 1: EDTA blood tube (8 mL),

Sample 2: Hirudin blood tubes (8 mL),

Sample 3: Heparin Lithium plasma tubes (8 mL),

Sample 4: Blood tube with clot activator for serum (8 mL),

Sample 5: PAXgene tube with RNAlater (2.5 mL).

Samples 1-3 are centrifuged immediately at 2000*g* for 10 minutes. The supernatant (plasma) is aliquoted in small volumes into 1.5 mL tubes.
The precipitate from sample 1 is aliquoted into 1.5 mL tubes.
Sample 4 is kept for 45-60 minutes at room temperature and is then centrifuged at 2000*g* for 10 minutes. The supernatant (serum) is aliquoted in small volumes into 1.5 mL tubes.
Subsequently, all the processed samples are transported in a - 20 °C freezer and stored in a central bio-banking facility at - 80 °C.
Sample 5 rests vertically at room temperature for 24-72 hours, is frozen at - 20 °C for 24-72 hours, and is subsequently stored at - 80 °C.

Cerebrospinal fluid (CSF) samples

Upon lumbar puncture, the first 1 mL of CSF obtained is discarded. Thereafter, 4-6 mL of CSF is obtained as feasible. Samples are immediately centrifuged at 2000*g*, and the supernatant is aliquoted into 1.5 mL tubes.

Muscle biopsies (Clinical trial 4)

After injection of local anaesthesia (Lidocaine 2%) one tru-cut biopsy is obtained containing 100 mg of skeletal muscle tissue from the lateral vastus muscle. The biopsy is transported to the Dept. of Neuropathology, Rigshospitalet for further processing.

Base-line information

The following demographic information is collected in ALS patients: Age, gender, subtype of disease, current stage of disease, T_0 (defined as the month and year of the first subjective symptoms that later led to the diagnosis of either probable ALS or definite ALS), treatment with riluzole, daily medication, and observed cognitive changes.

Data are pseudonymized and entered into an Excel-database.

An interview of all the ALS patients, based on the questionnaire ALS functional Rating Scale - Revised (ALSFRS-R) [9], is conducted by the primary investigator on the day of lumbar puncture and blood sampling (data collection day) and the score is noted in the database. (Figure 2)

Clinical phenotyping

The subtype of ALS is noted: sALS/fALS and the subtype of spinal, bulbar, or truncal ALS. Furthermore, it is noted whether the ALS specialists observe any sign of cognitive impairment. ALSFRS-R score, estimated on the day of data collection, is noted.

Patients are age categorized into age groups. Time of the first appearance of symptoms (T_0) is noted and the clinical status on data collection day is categorized.

Furthermore, we aim to develop and validate a new, simple, early progression score based on the ALSFRS-R score.

Analysis

Complement measurements

The concentrations of complement components (ficolin-1, ficolin-2, ficolin-3, collectin-11, pentraxin 3 (PTX3), mannose-binding lectin (MBL), mannose-

binding lectin/ficolin/CL-associated serine protease 3 (MASP-3), mannose-binding lectin/ficolin/Cl-associated protein 1 (MAP-1), complement activation products (C4c, C3bc, sC5b-9) as well as complement activation potentials (measured as activation on pathway-specific ligands: Human serum albumin/anti-human serum albumin immune complexes for classical pathway activation; lipopolysaccharide for alternative pathway activation; mannan for MBL-mediated lectin pathway activation; and acetylated bovine serum albumin for ficolin-mediated lectin pathway activation) are measured by enzyme-linked immunosorbent assays (ELISA) in plasma and CSF samples. The ELISA experiments are performed at the Laboratory of Molecular Medicine, Rigshospitalet, using specific monoclonal antibodies as previously described. [10-22]

Total serum concentrations of C4 and C3 are quantified by an automated turbidimetric protein analyser (SPAPLUS®, The Binding Site group LDT, Birmingham, UK) where sheep polyclonal antibodies against either human C3c or human C4 are applied (The Binding Site group LDT, Birmingham, UK).

Cytokine measurement

Selected cytokines as well as acute phase reactants will be analysed with a multiplex sandwich immunoassay with electrochemiluminescence: Plates, precoated with capture antibodies for the cytokines, are incubated with plasma samples. Subsequently, detection antibodies are put in the wells and then the plates are incubated. After washing, the detection levels are measured.

RNA expression and proteomics studies

Full blood is obtained in PAXgene® Blood RNA tubes with RNA later, an RNA stabilizing buffer, in order to be able to isolate RNA and make a gene expression

profile later on. At that point in time, we will be able to conduct proteomic analysis simultaneously as well as using the same samples of whole blood preserved in RNA later.

Neuropathological studies

For immunofluorescence staining, sections of ALS muscle biopsies from the lateral vastus muscle is air-dried and fixed in 4 % paraformaldehyde (PFA) at −20 °C. Slides are washed in phosphate-buffered saline (PBS), permeabilized in PBS/0.2 % TritonX, and blocked using PBS/5% fetal calf serum (FCS)/0.2 % TritonX. The sections are stained with primary antibodies directed against complement components and regulators (anti-C3c, anti-C1q, anti-C4c, anti-C5b-9, and anti-CD59) followed by incubation with fluorescence marked secondary antibodies. The motor end-plates are visualized by incubating with Alexa 488 conjugated anti-bungarotoxin, which binds to post-synaptic acetylcholine receptors on the muscle fibres, thus visualizing the end-plates. After staining, the sections are washed in PBS, air-dried, and mounted on slides. The muscle sections are analysed for complement staining and co-localizations with motor end-plates using confocal microscopy. Each motor end-plate identified on the surface of a muscle fibre will be counted using automated software, and the length of the end-plates will be measured in the ALS muscle biopsies. The size of end-plates will be measured and the number of immunoreactive areas per section will be scored.

Outcome measures

Primary and explorative outcomes

This is mainly an explorative study. We hypothesise that ALS is an autoinflammatory disease and that the complement system is implicated in the

pathophysiology of ALS. The primary outcome measures as specified for each substudy below will reflect the expected activation of the complement system.

Clinical trial 1

The primary outcome is the difference in haemolytic activity of plasma against healthy red erythrocytes as measured by absorbance between ALS patients and neurologically healthy controls. The difference in haemolytic activity between ALS patients and neurological controls is an exploratory outcome.

Clinical trial 2

The primary outcome is the activation potential of the ficolin-mediated lectin pathway. The activation potential of the classical pathway, the alternative pathway, and the MBL-mediated lectin pathway are exploratory outcomes.

Clinical trial 3

The primary outcome measure is the change in the plasma/CSF concentration ratio of ficolin-3 over time in patients with rapid compared to those with slow progression. The concentrations in plasma and CSF of ficolin-1, ficolin-2, ficolin-3, collectin-11, PTX3, MASP-3, MBL, MAP-1, C4c, C3bc, sC5b-9 as well as the plasma/CSF concentration ratios of ficolin-1, ficolin-2, collectin-11, PTX3, MASP-3, MBL, MAP-1, C3bc, C4c, and sC5b-9 are exploratory outcomes.

Clinical trial 4

The primary outcome is the presence of any marker of the complement system in the neuromuscular junction as visualised by confocal microscopy of muscle biopsies from ALS patients. These putative observations will be compared against an existing normative sample of muscle biopsies.

Sample size

Clinical trial 1

Overgaard et al measured the haemolytic activity after incubation of both healthy and ALS erythrocytes in both healthy plasma and ALS plasma. They described a mean difference of 0.20 (SE 0.052 in group of ALS patients, number 20, SD 0.22) in the absorbance (415 nm, incubation: 5 hrs) between ALS group and group of healthy controls. [23] At α=0.05 and β=0.20, we need to include 21 participants in each group. Taking into account possible dropouts, failed technical tests, etc., inclusion of 25 persons per group is planned.

Clinical trial 2

The number of subjects in each group is calculated at an α=0.05 and β=0.20. We compare the complement activation potential of three equal-sized groups. In healthy subjects, the complement activation potential is 100 % with a normal area ranging from 50-150 %, and the prevalence of subjects with a low complement activation potential (under 50 %) is under 10 %. If 100 subjects are included in each group, it will be possible to detect statistically significant differences between the groups corresponding to an odds ratio of 2.3, which would correspond to 20 % of ALS patients having a low complement activation potential caused by increased complement activity. [24]

Clinical trial 3

To our knowledge, no prior studies describe the activity of the complement system in ALS patients over time. This is hence a pilot study for which no sample size calculation has been made. We will include 20 ALS patients.

Clinical trial 4

This is a hypothesis-generating study as the presence of complement activity in living ALS patients have not been described previously; therefore, no sample size calculation has been made. We will include ten ALS patients.

Statistical analysis

Clinical trial 1

The haemolytic activity in the three groups (ALS patients, neurological controls, neurologically healthy controls) is compared using one-way ANOVA followed by t-tests to pinpoint differences between groups. Cut-off values and predictive values are calculated using receiver operating characteristic (ROC) curves.

Clinical trial 2

The concentration of complement markers and the complement activation potential are compared between ALS patients and the two control groups using stepwise, one-way ANOVA followed by t-tests, both are Bonferroni-corrected as appropriate. If necessary, we will log-transform the data to ensure a Gaussian distribution. The covariates, used for our step-wise, one-way ANOVA to test for differences between the three groups, are subject category (ALS patient, neurologically healthy control, or neurological control), gender, and age.
The ALS patients will be described and categorized by the covariates as illustrated in Table 1. We will analyse the covariates and the response variables by doing step-wise, one-way ANOVA to test for differences between the ALS subtypes.
Cut-off values and predictive values are calculated using receiver operating characteristic (ROC) curves.

Clinical trial 3

Analyses are carried out as described for clinical trial 2. In addition, we will conduct linear regression analyses with the levels of complement proteins as the dependent variable and time since onset of disease, gender, age, and subtype of ALS as explanatory variables.

Clinical trial 4

Complement deposition in the muscle fibres and in particular in the neuromuscular junctions is described qualitatively. The samples are scored by an investigator, who is blinded to clinical information, into either “Normal”, “Light degree”, and “Severe degree” of changes. These assessments will be compared with 2 x K tables and non-parametric statistics.

Discussion

We intend this bio-bank project to provide a novel starting point for future ALS research. Further study of ALS is of paramount importance for patients as there is currently no efficient treatment for this devastating and fatal disease. With the present project, a substantial amount of biological material from patients suffering from ALS will be obtained. We hope that the project will bring the ALS research a significant step forward, will inspire other groups to start similar projects regarding this and other rare diseases, and hence will enable future basic bio-bank research within this challenging field.

Trial limitations

Retrospectively, it has been common procedure to include patients with probable or definite ALS in clinical ALS research projects. However, some patients have symptoms of a slower progressing motor neuron disease and

therefore get the diagnosis of primary motor neuron disease. They will, however, eventually develop symptoms that meet the criteria for probable or definite ALS. Therefore, the inclusion criteria should probably be broadened to include these patients. [25]

All patients have been interviewed by the same person preventing inter-rater variability. The possibility of reporting bias is not prevented, however, as the interviewer might change the way of questioning over time, even though she or he is using the same questionnaire for all patients. In particular, the T_0 can be difficult for the patients to define and remember and lead to imprecise or even incorrect answers. [26]

Patients included in this study are expected to exhibit sizeable, inter-individual variability regarding the duration of symptoms, so it might be an advantage to include all potential ALS patients when they are first referred to the out-patient ALS clinics. It would then be possible to follow the patients as the symptoms progress.

Information about cognitive impairment/changes registered in the electronic patient file has been entered into the database. However, less severe symptoms in some cases of cognitive impairment might be overlooked by the clinicians. Alternatively, patients could undergo standardized neuropsychological testing.

The neurologically healthy controls in these clinical trials are included if they have no neurological symptoms based only on the anamnesis. However, to ensure that these are neurologically healthy, one could argue that all the neurologically healthy controls should have a clinical, neurological examination performed as well as an MRI scan of the CNS before inclusion.

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Author Contributions

KSO, SWP, AOL, KM, PG, and ALK conceived and designed the study. ALK drafted the protocol, and ALK, KSO, SWP, AOL, KP, KM, and PG revised the protocol for important intellectual content. ALK, AOL, KSO, and KM implemented the study at the clinical departments. ALK drafted the manuscript. All authors revised the manuscript for important intellectual content and assume responsibility for the final version.

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Competing interests

None declared

Patient consent for publication

Not required

Ethics approval and dissemination

The project has been approved by the Committees on Health Research Ethics in the Capital Region of Denmark (Approval number H-16017145) and the Danish Data Protection Agency (File number 2012-58-0004). Written informed consent is obtained from all participants. All results will be published in peer-reviewed, medical journals and further disseminated at international conferences.

Patient and public involvement

Patients or the public were not involved in the design, conduct, reporting, or dissemination plans of this research.

Data sharing

Data will be pseudonymised and will not be publicly available as per current Danish legislation. Data may, however, be available upon reasonable request and after approval by relevant authorities of a mutual data sharing agreement.

Steering committee

A steering committee will be established with representatives from clinical as well as basic ALS research groups in order to ensure easy access to the collected biological material for future, state-of-the-art ALS research projects.

Abbreviations:

ALS: Amyotrophic lateral sclerosis
ALSFRS-R: ALS functional rating scale revised
ANOVA: Analysis of variance
CNS: Central nervous system
CRF: Case report form
CSF: Cerebrospinal fluid
DAMPs: Damage associated molecular patterns
EDTA: Ethylenediamine tetraacetic acid
ELISA: Enzyme-linked immunosorbent assay
fALS: Familial amyotrophic lateral sclerosis
FCS: Fetal calf serum
g: the relative centrifugal force
MAP-1: Mannose-binding lectin/ficolin/Cl-associated protein 1
MASP-3: Mannose-binding lectin/ficolin/CL-associated serine protease 3
MRI: Magnetic resonance imaging
N: Number
NMJs: Neuromuscular junctions
PAMPs: Pathogen associated molecular patterns
PBS: Phosphate-buffered saline
PFA: Paraformaldehyde
PTX3: Pentraxin 3
RNA: Ribonucleid acid
ROC: Receiver operating characteristics
RT: Room temperature
SAH: Subarachnoid haemorrhage
sALS: Spontaneous amyotrophic lateral sclerosis
SD: Standard deviation
SE: Standard error
T_0: Time of first symptoms (month and year)

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Table 1: Overview and the inclusion status of the four substudies

Clinical trial 1: A case-control study: Haemolytic activity in ALS plasma	
Course of trial:	• Interviews • Blood samples • Separation of red blood cells and plasma • Red blood cells incubated with blood type matched plasma with *or* without inactivated complement system • The degree of haemolysis is measured and compared
Subjects: Aim/Included so far	ALS: 25/25 NC: 25/25 NHC: 25/25
Clinical trial 2: A case-control study: Collection of material for biobank and profiling of the complement system	
Course of trial:	• Interviews • Blood samples and cerebrospinal fluid by lumbar puncture • Biological material is prepared and freezed on site (-20 °C) • Transportation of samples to central biobank for storage (-80 °C) • Profiling of the complement system comparing the three groups
Subjects: Aim/Included so far	ALS: 100/96 NC: 100/61 NHC: 100/96
Clinical trial 3: A cohort study: Collection of material for biobank and a study of the complement system over time	
Course of trial:	
Subjects: Aim/Included so far	ALS: 20/0 NC: — NHC: —
Clinical trial 4: Pilot study: Searching for complement activity in the neuromuscular junctions of ALS patients	
Course of trial:	• Interviews • Tru-cut biopsy from dominant lateral vastus muscle • Transportation of muscle biopsy to Department of Neuropathology, Rigshospitalet • Analysis of complement activity in muscle fibers and neuromuscular junctions
Subjects: Aim/Included so far	ALS: 10/0 NC: — NHC: —

ALS: Amyotrophic lateral sclerosis; C: Celsius; NH: Neurological controls; NHC: Neurologically healthy controls.

Table 2: Demographic data of the included ALS patients in Clinical trial 2

Characterization of the ALS group (n: 96)

Inclusion site:	**n**	**%**
Glostrup Hospital	9	9%
Bispebjerg Hospital	33	34%
Roskilde Hospital	33	34%
Odense Hospital	16	17%
Aarhus Hospital	5	5%
Age (median age: 67):		
<40	3	3%
40-49	12	13%
50-59	13	14%
60-69	28	29%
>70	40	42%
Age when first symptoms occurred (median age: 65):		
<40	4	4%
40-49	13	14%
50-59	15	16%
60-69	35	36%
>70	29	30%
Gender:		
Female	40	42%
Male	56	58%
ALS subtype I:		
Familial ALS	2	2%
Spontaneous ALS	94	98%
ALS subtype II:		
Spinal ALS	60	63%
Bulbar ALS	28	29%
Both spinal and bulbar ALS	6	6%
Truncal ALS	2	2%
ALSFRS-R score on date collection day (Median score: 37):		
Mild ALS symptoms (ALSFRS-R score>36)	49	51%
Moderate ALS symptoms (24<ALSFRS-R score≤36)	33	34%
Severe ALS symptoms (ALSFRS-R score≤24)	14	15%
Progression rate estimation (Δtime*ALSFRS-R score on DC day):		
Slow progression rate	26	27%
Medium progression rate	37	39%
Aggressive progressive rate	33	34%
Overall survival time from onset of symptoms (Database update: 02-05-2020)		
Still alive (missing data)	39	41%
Short survival time (0-2 years)	22	23%
Medium survival time (2-4 years)	25	26%
Long survival time (4+ years)	10	10%
Cognitive impairments observed:		
Yes	21	22%
No	75	78%
Riluzole treatment on data collection day:		
Yes	33	34%
No	63	66%

ALS: Amyotrophic lateral sclerosis; ALSFRS-R: Amyotrophic lateral sclerosis functional rating scale – revised; N: Number

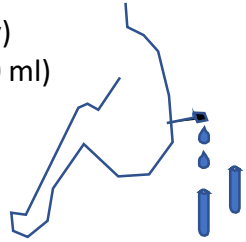
Legends to figures:

Fig. 1: Collection of biological material
C: Celsius; CSF: Cerebrospinal fluid; EDTA: Ethylenediamine tetraacetic acid; *g*: the relative centrifugal force; RNA: Ribonucleid acid.

Fig. 2: Inclusion and baseline registration
ALS: Amyotrophic lateral sclerosis; ALSFRS-R: Amyotrophic lateral sclerosis functional rating scale – revised; CT: Computerised tomography; NMD: Neuromuscular disease; SAH: Subarachnoid haemorrhage; T_0: Time of first symptoms (month and year).

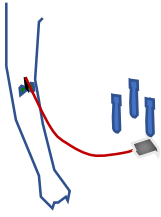
1. Cerebrospinal fluid (CSF) from lumbar puncture:

1 ml of CSF discarded
Routine CSF samples (if any)
Bio bank CSF samples (2-10 ml)

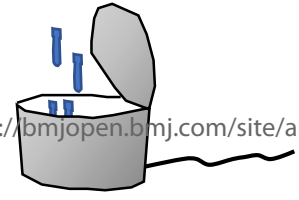


2. Blood samples from peripheral venous puncture:

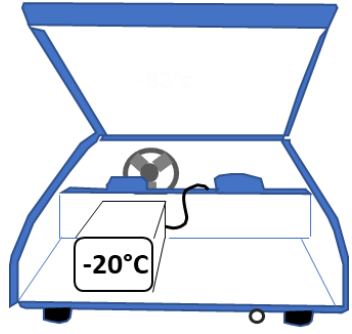
RNA later
Heparin plasma
EDTA plasma
EDTA precipitate
Hirudin plasma
Serum with clot activator



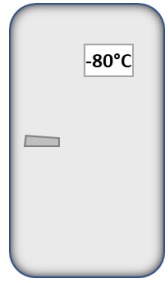
3. Centrifugation (2000*g*):



4. Transportation:



5. Biobank storage:



ALS patients

Recruitment of trial participants:

- Symptoms of motor neuron disease (NMD)
- Diagnosis of ”probable” or ”definite” ALS at outpatient ALS clinic

Base-line information on data collection day:

- Gender
- Age
- Concurrent diseases
- Daily medication
- T_0 (Appearance of first symptoms)
- ALSFRS-R
- Cognitive changes (if any)
- Subtype of ALS
- Onset site of first symptoms

Neurological controls

Recruitment of trial participants:

- Symptoms of SAH with normal CT scan *or*
- Admission to outpatient clinic due to symptoms of other neurological disease than NMD

Base-line information on data collection day:

- Gender
- Age
- Concurrent disease(s)
- Daily medication
- Admission diagnose(s)

Neurologically healthy controls

Recruitment of trial participants:

- No known neurological disease
- Spinal anaesthesia for elective orthopaedic surgery

Base-line information on data collection day:

- Gender
- Age
- Concurrent disease(s)
- Daily medication
- Surgical procedure performed

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Amyotrophic lateral sclerosis and the innate immune system: Protocol for establishing a biobank and statistical analysis plan

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Amyotrophic lateral sclerosis and the innate immune system

Protocol for establishing a biobank and statistical analysis plan

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Abstract

Introduction: Amyotrophic lateral sclerosis (ALS) is a devastating, progressive disease that causes degeneration of the motor neurons leading to paresis of the bulbar and the skeletal musculature. The pathogenesis of ALS remains unknown. We will test the hypothesis that the complement system is involved in the pathophysiology of ALS. This protocol article describes our efforts to establish a national Danish ALS biobank. The primary aim is to obtain biological material from ALS patients for the current study as well as for future studies.

Methods and analysis: We intend to establish an observational ALS bio-bank; some of the material from this biobank will be used for a prospective, observational case-control study. The participants are ALS patients, neurologically healthy controls, and non-ALS neurological controls. Each participant consents to be interviewed and to donate blood and cerebrospinal fluid to the biobank. Analysis of the complement system will be carried out on the three groups of patients and compared.

Ethics and dissemination: The project has been approved by the Committees on Health Research Ethics in the Capital Region of Denmark (Approval number H-16017145) and the Danish Data Protection Agency (File number 2012-58-0004). All results will be published in peer-reviewed, medical journals and presented at scientific conferences.

Trial registration: Clinicaltrial.gov, protocol ID: NCT02869048, initial release: 06/28/2016

Keywords: Amyotrophic lateral sclerosis, complement system, bio-bank, protocol, innate immune system, case-control study

Article summary

Strengths and limitations of this study

- This prospective, observational study ensures a national ALS biobank which will facilitate many future clinical studies of a rare disease such as ALS.
- Even though ALS is a heterogenous disease, all patients face a rapid deterioration and severe distress and the design of this study provides recruitment of ALS patients at an early clinical stage hence facilitating trial participation.
- The diagnosis of ALS is difficult, and patients follow highly variable courses of disease; thus some patients who have been recruited for the study will eventually be diagnosed with other diseases and need to be excluded from the present study; conversely, some patients who are in the early phase of ALS may be missed because of atypical symptoms and will therefore be recruited in a later phase.

Introduction

Amyotrophic lateral sclerosis (ALS) is an aggressive disease that causes progressive degeneration of the upper and lower motor neurons leading to severe muscular dystrophy, fasciculations, hyperreflexia, and paresis of the bulbar and skeletal musculature. As the disease inevitably progresses, the patient becomes unable to move, speak, swallow, and breathe. In addition to the neuromuscular symptoms, frontotemporal dementia appears in up to 15 % of all ALS patients.[1]

ALS is a rare disease with an incidence of 1-2/100.000 and a prevalence of 4-6/100.000. In 70% of all ALS cases, initial symptoms appear in the upper or

lower extremities (spinal ALS), in five percent symptoms initially appears in the truncus (truncal ALS), and in 25% a bulbar onset (bulbar ALS) is seen. In a systematic review of the global epidemiology of ALS, the median survival time was reported as 24-48 months from symptom onset.[2]

Five to ten percent of all ALS cases are of the familial type (fALS) which is genetically heterogeneous mainly with a Mendelian inheritance pattern.[3] In the remaining patients, there is no family history (sporadic ALS (sALS)).[3]

The pathogenesis of ALS remains unknown and presently, there is no effective treatment to stop the progression. The immune system has been hypothesized to be an important, pathophysiological factor. This is based on the fact that immunological reactions appear both in the proximity of the degenerating motor neurons in the central nervous system (CNS) and systemically.[4]

The complement system plays a central role in the innate immune system. The complement system initiates inflammation and activates innate as well as adaptive immune cells by detecting invading pathogens or damaged host cells. According to recent animal models of ALS, the very first detectable pathophysiological changes are seen in the neuromuscular junctions and might be a sign of aberrant complement activation.[5 6] Such inappropriate activation of the complement cascades may participate in the initiation of the destruction of the neuromuscular junctions by an autoinflammatory mechanism. Furthermore, aberrant complement activation has been found in the proximity of the degenerating motor neurons during ALS progression. In addition, findings of complement activation in the blood and in the muscle tissue, as well as an unexplained erythrocyte cytotoxicity phenomenon found in the plasma of ALS

patients, suggest that the complement system could play an important role in the pathophysiology during the progression of ALS.[4 7]

To be able to develop an effective medical treatment, it is essential to study the pathogenesis and the pathophysiology of ALS. Since complement inhibition employing the terminal complement inhibitor ravulizumab now is entering a phase 3 clinical trial for the treatment of ALS, it is crucial to firmly establish how the complement system affects the progression of ALS (https://news.alexionpharma.com). Understanding the pathophysiology of the disease provides a tool to develop diagnostic methods such as biomarkers. This may optimise the diagnostic process ensuring a faster and more precise diagnosis. In addition, this could increase the knowledge of the heterogeneity of the ALS symptoms.

Aim

The overall, long-term objective of this project is to establish an ALS bio-bank to facilitate future basic ALS research. Under the assumption that ALS is an autoinflammatory disease, another aim of the project is to disclose whether complement activation plays an essential role in the pathophysiology of ALS. The protocol describes the design and data collection methods in detail in order to inform other researchers of the bio-bank.

Methods

Design

This Danish national ALS bio-bank project is designed as a prospective, observational case-control study, based on the Standard Protocol Items:

Recommendations for Interventional Trials (SPIRIT) guidelines.[8] The bio-bank will be established to facilitate future studies. The first study comprises four substudies (Substudy 1-4, Table 1) of which Substudy 1 and 2 are ongoing and the recruitment of participants for Substudy 3 and 4 is planned to begin in the fall, 2020. Collection of biological material from ALS patients for the longitudinal cohort study, Substudy 3, is planned to continue as long as the included ALS patients consent to lumbar puncture, blood sampling, and a short interview every six months, if possible.

Patient and public involvement

Patients or the public were not involved in the design, conduct, reporting, or dissemination plans of this research.

Participants

The national ALS bio-bank comprises samples from three groups of patients: ALS patients, neurologically healthy patients (healthy controls), and patients with neurological diseases other than ALS (neurological controls). All the participants included are above 18 yrs.

Patients with ALS*:* Patients referred to an ALS out-patient clinic at either of five Danish hospitals (Table 2) due to symptoms suspected to be early symptoms of ALS are informed and invited to participate in the project during the standard clinical workup. If a patient at the end of the planned workup is diagnosed with either probable or definite ALS according to the El Escorial Revised criteria [9] and consents to inclusion, the patient is included in the project. Furthermore, *all probable or definite ALS patients* who have already been permanently associated

to an ALS outpatient clinic (post clinical investigation) and who accept to undergo an additional lumbar puncture are invited to be part of the project.

Neurologically healthy controls (NHCs): Neurologically healthy patients scheduled for elective orthopaedic surgery under spinal anaesthesia are invited to participate.

Neurological controls (NCs): Patients admitted to hospital due to acute symptoms consistent with a subarachnoid haemorrhage (SAH) are all subjected to a CT scan of the head. If this scan is without any signs of haemorrhage, an acute lumbar puncture will be carried out. We recruit participants for the neurological control group from this group of patients and the primary investigator performs the lumbar puncture. Furthermore, patients who are referred for a planned lumbar puncture as part of a clinical neurological investigation due to neurological symptoms are also recruited for the NC group.

Exclusion criteria for the two control groups: Subjects with motor neuron disease, chronic inflammatory, or autoimmune diseases are excluded from the neurologically healthy control group and the neurological control group.

Settings

ALS patients are recruited from five ALS out-patient clinics at five Danish neurological departments: Bispebjerg Hospital, Rigshospitalet (Glostrup) (both University of Copenhagen), Roskilde University Hospital, Odense University Hospital, and Aarhus University Hospital. Neurologically healthy controls are recruited at Gildhøj Private Hospital, Denmark. Controls from the neurological group are recruited at the neurological department at Rigshospitalet (Glostrup), Denmark. At each hospital, one local investigator is appointed. During sample collection, the primary investigator is present to obtain biological material from each patient and to process and freeze the samples using a portable “mini-lab”.

All data collected on case report forms are anonymised, gathered, and stored in an audited, central, electronic database. All biological material is transported to the Department of Clinical Immunology at Rigshospitalet, Denmark to be stored in bio-banking facilities.

Data collection

In ALS patients and patients in the neurological control group, blood samples are drawn from a peripheral vein after the performance of the lumbar puncture. In neurologically healthy controls, for logistical reasons, the blood samples are drawn before the lumbar puncture. All the samples are processed by the primary investigator at the respective inclusion site and are transported in a - 20 °C freezer to a central bio-bank where they are stored at - 80 °C. (Figure 1)

Biological samples

Blood samples

Venous blood samples are collected by the primary investigator or by a skilled clinical nurse. Blood is collected in Sample 1: EDTA blood tube (8 mL), Sample 2: Hirudin blood tubes (8 mL), Sample 3: Heparin Lithium plasma tubes (8 mL), Sample 4: Blood tube with clot activator for serum (8 mL), Sample 5: PAXgene tube with RNAlater (2.5 mL). Samples 1-3 are centrifuged immediately at 2000*g* for 10 minutes. The supernatant (plasma) is aliquoted in small volumes into 1.5 mL tubes. The precipitate from sample 1 is aliquoted into 1.5 mL tubes. Sample 4 is kept for 45-60 minutes at room temperature and is then centrifuged at 2000*g* for 10 minutes. The supernatant (serum) is aliquoted in small volumes into 1.5 mL tubes. All samples are processed by the primary investigator. Subsequently, all the processed samples are transported in a - 20 °C freezer and stored in a central bio-banking facility at - 80 °C. Sample 5 is placed vertically at

room temperature for 24-72 hours, is frozen at - 20 °C for 24-72 hours, and is subsequently stored at - 80 °C.

Cerebrospinal fluid (CSF) samples

Upon lumbar puncture, the first 1 mL of CSF obtained is discarded. Thereafter, 4-6 mL of CSF is obtained as feasible. Samples are immediately centrifuged at 2000*g*, and the supernatant is aliquoted into 1.5 mL tubes.

Bio-bank facility

The processed blood samples and the processed CSF samples will be stored in a bio-bank in a - 80 °C freezer located at Laboratory of Molecular Medicine, Department of Clinical Immunology, Rigshospitalet, Copenhagen, Denmark.

Muscle biopsies (Substudy 4)

After injection of local anaesthesia (Lidocaine 2%) one tru-cut biopsy is obtained containing 100 mg of skeletal muscle tissue from the lateral vastus muscle. The biopsy is transported to the Department of Neuropathology, Rigshospitalet for further processing.

Base-line information

The following demographic information about the ALS patients are collected: Age, gender, subtype of disease, current stage of disease, T_0 (defined as the month and year of the first subjective symptoms that later led to the diagnosis of either probable ALS or definite ALS), treatment with riluzole, daily medication, and observed cognitive changes. Data are pseudonymized and entered into an Excel-database only accessible for ALK, PG, and KM and the database is updated, stored, and logged in accordance with Danish law and regulations set by the

Danish Data Protection Agency. An interview of all the ALS patients, based on the questionnaire ALS functional Rating Scale - Revised (ALSFRS-R)[10], is conducted by the primary investigator on the day of lumbar puncture and blood sampling (data collection day) and the score is noted in the database. (Figure 2)

Clinical phenotyping

The subtype of ALS is noted: sALS/fALS and the subtype of spinal, bulbar, or truncal ALS. Furthermore, it is noted whether the ALS specialists observe any sign of cognitive impairment. ALSFRS-R score, estimated on the day of data collection, is noted. Patients are age categorized into age groups. Time of the first appearance of symptoms (T_0) is noted and the clinical status on data collection day is categorized.

Analysis

Complement measurements

The concentrations of complement components (ficolin-1, ficolin-2, ficolin-3, collectin-11, pentraxin 3 (PTX3), mannose-binding lectin (MBL), mannose-binding lectin/ficolin/CL-associated serine protease 3 (MASP-3), mannose-binding lectin/ficolin/Cl-associated protein 1 (MAP-1), complement activation products (C4c, C3bc, sC5b-9) as well as complement activation potentials (measured as activation on pathway-specific ligands: Human serum albumin/anti-human serum albumin immune complexes for classical pathway activation; lipopolysaccharide for alternative pathway activation; mannan for MBL-mediated lectin pathway activation; and acetylated bovine serum albumin for ficolin-mediated lectin pathway activation) are measured by enzyme-linked immunosorbent assays (ELISA) in plasma and CSF samples. The ELISA experiments are performed at the Laboratory of Molecular Medicine,

Rigshospitalet, using specific monoclonal antibodies as previously described.[11-23] Total serum concentrations of C4 and C3 are quantified by an automated turbidimetric protein analyser (SPAPLUS®, The Binding Site group LDT, Birmingham, UK) where sheep polyclonal antibodies against either human C3c or human C4 are applied (The Binding Site group LDT, Birmingham, UK).

Cytokine measurement

Selected cytokines as well as acute phase reactants will be analysed with a multiplex sandwich immunoassay with electrochemiluminescence: Plates, precoated with capture antibodies for the cytokines, are incubated with plasma samples. Subsequently, detection antibodies are put in the wells and then the plates are incubated. After washing, the detection levels are measured.

RNA expression and proteomics studies

Full blood is obtained in PAXgene® Blood RNA tubes with RNA later, an RNA stabilizing buffer, for future isolation of RNA and gene expression profiling. At that point in time, we will be able to conduct proteomic analysis simultaneously as well as using the same samples of whole blood preserved in RNA later.

Neuropathological studies

For immunofluorescence staining, sections of ALS muscle biopsies from the lateral vastus muscle is air-dried and fixed in 4 % paraformaldehyde (PFA) at −20 °C. Slides are washed in phosphate-buffered saline (PBS), permeabilized in PBS/0.2 % TritonX, and blocked using PBS/5% fetal calf serum (FCS)/0.2 % TritonX. The sections are stained with primary antibodies directed against complement components and regulators (anti-C3c, anti-C1q, anti-C4c, anti-C5b-9, and anti-CD59) followed by incubation with fluorescence marked secondary

antibodies. The motor end-plates are visualized by incubating with Alexa 488 conjugated anti-bungarotoxin, which binds to post-synaptic acetylcholine receptors on the muscle fibres, thus visualizing the end-plates. After staining, the sections are washed in PBS, air-dried, and mounted on slides. The muscle sections are analysed for complement staining and co-localizations with motor end-plates using confocal microscopy. Each motor end-plate identified on the surface of a muscle fibre will be counted using automated software, and the length of the end-plates will be measured in the ALS muscle biopsies. The size of end-plates will be measured and the number of immunoreactive areas per section will be scored.

Outcome measures, sample size, and statistics for each substudy

Substudy 1

Primary and explorative outcomes

The primary outcome is the difference in haemolytic activity of plasma against healthy red erythrocytes as measured by absorbance between ALS patients and neurologically healthy controls. The difference in haemolytic activity between ALS patients and neurological controls is an exploratory outcome.

Sample size

Overgaard et al measured the haemolytic activity after incubation of healthy as well as ALS erythrocytes in both healthy plasma and ALS plasma. They described a mean difference of 0.20 (SE 0.052 in the group of ALS patients, number 20, SD 0.22) in the absorbance (415 nm, incubation: 5 hrs) between ALS group and group of healthy controls.[24] At $\alpha=0.05$ and $\beta=0.20$, we need to include 21

participants in each group. Taking into account possible dropouts, failed technical tests, etc., inclusion of 25 persons per group is planned.

Statistical analysis

The haemolytic activity in the three groups (ALS patients, neurological controls, neurologically healthy controls) will be compared using one-way ANOVA followed by t-tests to pinpoint differences between groups. Cut-off values and predictive values will be calculated using receiver operating characteristic (ROC) curves.

Substudy 2

Primary and explorative outcomes

The primary outcome is the activation potential of the ficolin-mediated lectin pathway. The activation potential of the classical pathway, the alternative pathway, and the MBL-mediated lectin pathway are exploratory outcomes.

Sample size

The number of subjects in each group is calculated using an α=0.05 and β=0.20. We compare the complement activation potential of three equal-sized groups. In healthy subjects, the complement activation potential is 100 % with a normal area ranging from 50-150 %, and the prevalence of subjects with a low complement activation potential (under 50 %) is under 10 %. If 100 subjects are included in each group, it will be possible to detect statistically significant differences between the groups corresponding to an odds ratio of 2.3, which would correspond to 20 % of ALS patients having a low complement activation potential caused by increased complement activity.[25]

Statistical analysis

The concentration of complement markers and the complement activation potential will be compared between ALS patients and the two control groups using stepwise ANOVA followed by t-tests, Bonferroni-corrected as appropriate. If necessary, we will log-transform the data to ensure a Gaussian distribution. The covariates, that will be used for the step-wise ANOVA to test for differences between the three groups, are subject category (ALS patient, neurologically healthy control, or neurological control), gender, and age.

The ALS patients will be described and categorized by the covariates as illustrated in Table 1. We will analyse the covariates and the response variables by doing step-wise, one-way ANOVA to test for differences between the ALS subtypes.

Cut-off values and predictive values are calculated using receiver operating characteristic (ROC) curves.

Substudy 3

Primary and explorative outcomes

The primary outcome measure is the change in the plasma/CSF concentration ratio of ficolin-3 over time in patients with rapid progression compared to those with slow progression. The concentrations in plasma and CSF of ficolin-1, ficolin-2, ficolin-3, collectin-11, PTX3, MASP-3, MBL, MAP-1, C4c, C3bc, sC5b-9 as well as the plasma/CSF concentration ratios of ficolin-1, ficolin-2, collectin-11, PTX3, MASP-3, MBL, MAP-1, C3bc, C4c, and sC5b-9 are exploratory outcomes.

Sample size

To our knowledge, no prior studies describe the activity of the complement system in ALS patients over time. This is hence a pilot study for which no sample size calculation has been made. We will include 20 ALS patients.

Statistical analysis

Analyses are carried out as described for Substudy 2. In addition, we will conduct linear regression analyses with the levels of complement proteins as the dependent variable and time since onset of disease, gender, age, and subtype of ALS as explanatory variables.

Substudy 4

Primary and explorative outcomes

The primary outcome is the presence of any marker of the complement system in the neuromuscular junction as visualised by confocal microscopy of muscle biopsies from ALS patients. These putative observations will be compared against an existing normative sample of muscle biopsies.

Sample size

This is a hypothesis-generating study as the presence of complement activity in living ALS patients have not been described previously; therefore, no sample size calculation has been made. We will include ten ALS patients.

Statistical analysis

Complement depositions in the muscle fibres and in particular in the neuromuscular junctions are described qualitatively. The samples are scored by an investigator, who is blinded to clinical information, into either “Normal”,

"Light degree", or "Severe degree" of changes. These assessments will be compared with 2 x K tables and non-parametric statistics.

Discussion

ALS is a rapidly progressive, fatal disease. Despite the aggressive course and devastating consequences, there is currently no effective treatment of ALS. This manuscript describes the generation of a Danish ALS bio-bank that will hopefully provide a starting point for future national ALS research. With the present project, a substantial amount of biological material from patients suffering from ALS will be obtained. We hope that the project will bring ALS research a significant step forward, will inspire other groups to start similar projects regarding this and other rare diseases, and hence will enable future basic bio-bank research within this challenging field.

The generation of this bio-bank was inspired by the hypothesis that the innate immune system plays a significant role in the pathogenesis or the pathophysiology of ALS. With a specific aim to profile the complement system in ALS patients, it became important to ensure that the two control groups do not include patients with diseases with neither primary nor secondary complement activation even if this means that the two control groups will not be comparative in age and gender.

Several initiatives have conducted multi-center, interventional clinical trials and established large ALS bio-banks.[26] Continuing the collection of biological material from patients with this rare disease will maintain and expand the capacity to study the increasing number of hypotheses generated by the spatiotemporal studies conducted on animal ALS models.[5 6 27 28] With the present protocol, we intend to obtain biological material from ALS patients starting in a

relatively early phase of clinical disease. Furthermore, ALS patients are invited to participate in the cohort study (Substudy 3) which will add a spatiotemporal aspect to the bio-bank.

All Danish citizens are registered in the National Patient Registry (DNPR) which is one of the oldest complete, national patient registries in the world. DNPR is linkable on an individual level with other clinical and administrative registries.[29] Hence, this poses a unique possibility to conduct registry-based research combined with data from this ALS biobank in future studies.

More than 20 genetic mutations have been associated with ALS, the most well-known and well researched mutations being the *SOD1*, *FUS*, *TARDBP*, and *C9ORF72* genes.[30] Even though ALS seems to have a multifactorial aetiology, genetic mutations may play a pivotal role in the pathogenesis of ALS. Our planned substudies do not include genetic analysis. However, future genetic studies will be possible as biological material in the form of EDTA precipitate will be secured for the bio-bank.

Trial limitations

All patients have been interviewed by the same person preventing inter-rater variability. The possibility of reporting bias is not prevented, however, as the interviewer might subtly change the way of questioning over time, even though they are using the same questionnaire for all patients. Additionally, the T_0 can be difficult for the patients to define and remember and lead to imprecise or even incorrect answers.[31]

Retrospectively, it has been common procedure to include patients with probable or definite ALS in clinical ALS research projects. Some patients have slowly progressing symptoms of a motor neuron disease which initially do not

meet the criteria of probable or definite ALS as the upper and lower motor neurons are not yet both affected. Even so, the symptoms may progress in which case the patient will eventually meet the criteria of probable or definite ALS. With the inclusion criteria stated for the present protocol, we realise that we may miss inclusion of this subset of (possible) ALS patients and hence the opportunity to observe the initial phase of such patients.[32]

Information about cognitive impairment/changes registered in the electronic patient file has been entered into the database. However, less severe symptoms in some cases of cognitive impairment might be overlooked by the clinicians, and ideally, patients should undergo standardized, neuropsychological testing. However, as it was not within the scope of this project to ensure neuropsychological testing of the ALS patients, we elected to rely on the practice of the clinical inclusion sites, which unfortunately does not always comprise testing, regardless of cognitive changes.

Substudy 4 is a pilote study based on tru-cut skeletal muscle biopsies from ten ALS patients. Since only one biopsy is obtained from each patient, this is a rather small amount of tissue in particular for a neuromuscular-junction study. We chose this option as a compromise given the pilot nature of the study, to inflict as little pain as possible on the patient.

The neurologically healthy controls in the bio-bank and hence also for the four substudies are included if they have no neurological symptoms based only on the anamnesis. However, to ensure that these are neurologically healthy, one could argue that all the neurologically healthy controls should have a clinical neurological examination performed as well as an MRI scan of the CNS before inclusion.

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Author Contributions

KSO, SWP, AOL, KM, PG, and ALK conceived and designed the study. ALK drafted the protocol, and ALK, KSO, SWP, AOL, KP, KM, and PG revised the protocol for important intellectual content. ALK, AOL, KSO, and KM implemented the study at the clinical departments. ALK drafted the manuscript. All authors revised the manuscript for important intellectual content and assume responsibility for the final version.

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Competing interests

None declared

Patient consent for publication

Not required

Ethics approval and dissemination

The project has been approved by the Committees on Health Research Ethics in the Capital Region of Denmark (Approval number H-16017145) and the Danish Data Protection Agency (File number 2012-58-0004). Written informed consent is obtained from all participants. All results will be published in peer-reviewed, medical journals and further disseminated at international conferences.

Data sharing

Data will be pseudonymised and will not be publicly available as per current Danish legislation. Data may, however, be available upon reasonable request and after approval by relevant authorities of a mutual data sharing agreement.

Steering committee

A steering committee will be established with representatives from clinical as well as basic ALS research groups in order to ensure easy access to the collected biological material for future, state-of-the-art ALS research projects.

Abbreviations:

ALS: Amyotrophic lateral sclerosis
ALSFRS-R: ALS functional rating scale revised
ANOVA: Analysis of variance
CNS: Central nervous system
CRF: Case report form
CSF: Cerebrospinal fluid
CT: Computerised tomography
DAMPs: Damage associated molecular patterns
DNPR: Danish National Patient Registry
EDTA: Ethylenediamine tetraacetic acid
ELISA: Enzyme-linked immunosorbent assay

fALS: Familial amyotrophic lateral sclerosis
FCS: Fetal calf serum
g: the relative centrifugal force
MAP-1: Mannose-binding lectin/ficolin/Cl-associated protein 1
MASP-3: Mannose-binding lectin/ficolin/CL-associated serine protease 3
MRI: Magnetic resonance imaging
N: Number
NCs: Neurological controls
NHCs: Neurologically healthy controls
NMD: Neuromuscular disease
PAMPs: Pathogen associated molecular patterns
PBS: Phosphate-buffered saline
PFA: Paraformaldehyde
PTX3: Pentraxin 3
RNA: Ribonucleid acid
ROC: Receiver operating characteristics
RT: Room temperature
SAH: Subarachnoid haemorrhage
sALS: Sporadic amyotrophic lateral sclerosis
SD: Standard deviation
SE: Standard error
SPIRIT: Standard protocol items: Recommendations for interventional trials
T_0: Time of first symptoms (month and year)

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Table 1: Overview and the inclusion status of the four substudies

Substudy 1: A case-control study: Haemolytic activity in ALS plasma	
Course of study:	• Interviews • Blood samples • Separation of red blood cells and plasma • Red blood cells incubated with blood type matched plasma with *or* without inactivated complement system • The degree of haemolysis is measured and compared
Subjects: Aim/Included so far	ALS: 25/25 NC: 25/25 NHC: 25/25
Substudy 2: A case-control study: Collection of material for biobank and profiling of the complement system	
Course of study:	• Interviews • Blood samples and cerebrospinal fluid by lumbar puncture • Biological material is prepared and freezed on site (-20 °C) • Transportation of samples to central biobank for storage (-80 °C) • Profiling of the complement system comparing the three groups
Subjects: Aim/Included so far	ALS: 100/98 NC: 100/61 NHC: 100/96
Substudy 3: A cohort study: Collection of material for biobank and a study of the complement system over time	
Course of study:	
Subjects: Aim/Included so far	ALS: 20/0 NC: — NHC: —
Substudy 4: Pilot study: Searching for complement activity in the neuromuscular junctions of ALS patients	

Course of study:	• Interviews • Tru-cut biopsy from dominant lateral vastus muscle • Transportation of muscle biopsy to Department of Neuropathology, Rigshospitalet • Analysis of complement activity in muscle fibers and neuromuscular junctions
Subjects: Aim/Included so far	ALS: 10/0 NC: — NHC: —

ALS: Amyotrophic lateral sclerosis; C: Celsius; NC: Neurological controls; NHC: Neurologically healthy controls.

Table 2: Demographic data of the included ALS patients in Substudy 2

Characterization of the ALS group (n: 96)

Inclusion site:	**n**	**%**
Glostrup Hospital	9	9%
Bispebjerg Hospital	33	34%
Roskilde Hospital	33	34%
Odense Hospital	16	17%
Aarhus Hospital	5	5%
Age (median age: 67):		
<40	3	3%
40-49	12	13%
50-59	13	14%
60-69	28	29%
>70	40	42%
Age when first symptoms occurred (median age: 65):		
<40	4	4%
40-49	13	14%
50-59	15	16%
60-69	35	36%
>70	29	30%
Gender:		
Female	40	42%
Male	56	58%
ALS subtype I:		
Familial ALS	2	2%

Spontaneous ALS	94	98%
ALS subtype II:		
Spinal ALS	60	63%
Bulbar ALS	28	29%
Both spinal and bulbar ALS	6	6%
Truncal ALS	2	2%
ALSFRS-R score on date collection day (Median score: 37):		
Mild ALS symptoms (ALSFRS-R score>36)	49	51%
Moderate ALS symptoms (24<ALSFRS-R score≤36)	33	34%
Severe ALS symptoms (ALSFRS-R score≤24)	14	15%
Progression rate estimation (Δtime*ALSFRS-R score on DC day):		
Slow progression rate	26	27%
Medium progression rate	37	39%
Aggressive progressive rate	33	34%
Overall survival time from onset of symptoms (Database update: 02-05-2020)		
Still alive (missing data)	39	41%
Short survival time (0-2 years)	22	23%
Medium survival time (2-4 years)	25	26%
Long survival time (4+ years)	10	10%
Cognitive impairments observed:		
Yes	21	22%
No	75	78%
Riluzole treatment on data collection day:		
Yes	33	34%
No	63	66%

ALS: Amyotrophic lateral sclerosis; ALSFRS-R: Amyotrophic lateral sclerosis functional rating scale – revised; N: Number

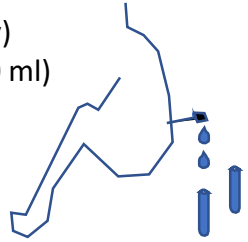
Legends to figures:

Fig. 1: Collection of biological material
C: Celsius; CSF: Cerebrospinal fluid; EDTA: Ethylenediamine tetraacetic acid; *g*: the relative centrifugal force; RNA: Ribonucleid acid.

Fig. 2: Inclusion and baseline registration
ALS: Amyotrophic lateral sclerosis; ALSFRS-R: Amyotrophic lateral sclerosis functional rating scale – revised; CT: Computerised tomography; NMD: Neuromuscular disease; SAH: Subarachnoid haemorrhage; T_0: Time of first symptoms (month and year).

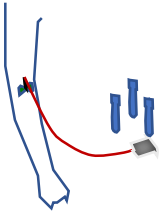
1. Cerebrospinal fluid (CSF) from lumbar puncture:

1 ml of CSF discarded
Routine CSF samples (if any)
Bio bank CSF samples (2-10 ml)

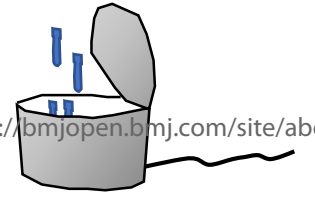


2. Blood samples from peripheral venous puncture:

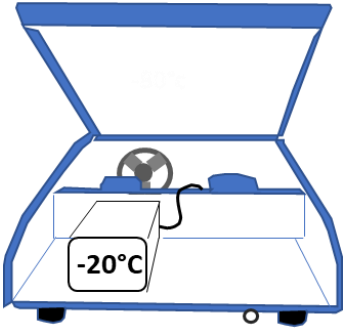
RNA later
Heparin plasma
EDTA plasma
EDTA precipitate
Hirudin plasma
Serum with clot activator



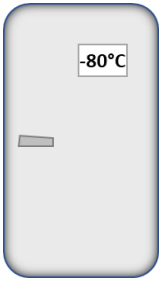
3. Centrifugation (2000*g*):



4. Transportation:



5. Biobank storage:



ALS patients

Recruitment of trial participants:

- Symptoms of motor neuron disease (NMD)
- Diagnosis of ”probable” or ”definite” ALS at outpatient ALS clinic

Base-line information on data collection day:

- Gender
- Age
- Concurrent diseases
- Daily medication
- T_0 (Appearance of first symptoms)
- ALSFRS-R
- Cognitive changes (if any)
- Subtype of ALS
- Onset site of first symptoms

Neurological controls

Recruitment of trial participants:

- Symptoms of SAH with normal CT scan *or*
- Admission to outpatient clinic due to symptoms of other neurological disease than NMD

Base-line information on data collection day:

- Gender
- Age
- Concurrent disease(s)
- Daily medication
- Admission diagnose(s)

Neurologically healthy controls

Recruitment of trial participants:

- No known neurological disease
- Spinal anaesthesia for elective orthopaedic surgery

Base-line information on data collection day:

- Gender
- Age
- Concurrent disease(s)
- Daily medication
- Surgical procedure performed